Glial response to 17β-estradiol in neonatal rats with excitotoxic brain injury

Running title: E2 and neonatal brain injury

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Abstract

White-matter injury is the most common cause of the adverse neurodevelopmental outcomes observed in preterm infants. Only few options exist to prevent perinatal brain injury associated to preterm delivery. 17β-estradiol (E2) is the predominant estrogen in circulation and has been shown to be neuroprotective in vitro and in vivo. However, while E2 has been found to modulate inflammation in adult models of brain damage, how estrogens influence glial cells response in the developing brain needs further investigations. Using a model of ibotenate-induced brain injury, we have refined the effects of E2 in the developing brain. E2 provides significant neuroprotection both in the cortical plate and the white matter in neonatal rats subjected to excitotoxic insult mimicking white matter and cortical damages frequently observed in very preterm infants. E2 promotes significant changes in microglial phenotypes balance in response to brain injury and the acceleration of oligodendrocyte maturation. Maturational effects of E2 on myelination process were observed both in vivo and in vitro. Altogether, these data demonstrate that response of glial cells to E2 could be responsible for its neuroprotective properties in neonatal excitotoxic brain injury.

Introduction

Preterm birth is a common condition that affects roughly 1 in every 10 pregnancies, including in developed countries. In 2010, the Global Burden of Disease Study estimated that preterm birth was the most frequent cause of death and disability in children under the age of 5 years (Liu et al., 2012). Indeed, in addition to the immediate problem posed by the care of preterm infants and complications due to the immaturity of their organs, in 25-50% of them, prematurity is associated with long-term neurological disability, cerebral palsy, cognitive impairment and behavioral deficits, presenting enormous challenges to individuals, their families and society. This high rate of disability is partly a consequence of the improved survival of more and more immature infants, thanks to innovations in perinatal medicine over the past 30 years (Marlow et al., 2005). There are currently few treatment options to prevent brain injury associated to preterm delivery (Favrais et al., 2014). The need for novel strategies and care practices to curb the neurodevelopmental sequelae following premature birth is thus urgent.

White-matter injury is the most common cause of the adverse neurodevelopmental outcomes observed in preterm infants (Back et al., 2014). In neonatal rodents, excitotoxic injury using glutamate agonists mimics some aspects of the diffuse white and grey matter abnormalities, collectively known as encephalopathy of prematurity, observed in the brain of human preterm neonates (Gressens et al., 1996; Volpe, 2009). Recent evidence suggests the existence of crosstalk between oligodendrocytes and their surroundings, including microglial cells and the hormonal environment. The injury-mediated activation of microglial cells plays a key role in disrupting the developmental maturation of the oligodendroglial lineage (Kaindl et al., 2009), and potentiates white matter injury and cognitive dysfunction into adulthood (Moretti et al., 2015).

Estrogens are lipophilic steroid hormones that can diffuse across the blood-brain barrier. However, the role of estrogens is not limited to the maintenance of female reproductive function and they have remarkable effects at various concentrations on several systems including the cardiovascular, immune, and nervous systems (Arevalo et al., 2015). 17β-estradiol (E2), an isomer of estradiol, is the predominant estrogen in circulation and has been shown to be neuroprotective in vitro and in vivo in various models of brain damage (Arevalo et al., 2010; Mc Carthy, 2008; Gerstner et al., 2007; Gerstner et al., 2009). E2 directly promotes cell survival and synaptic plasticity, prevents axonal and dendritic pruning, and modulates levels of neurotransmitters and their receptors, leading to improved cell survival and neurite branching. However, while E2 has been found to modulate inflammation in adult models of brain damage, whether estrogens also influence the crosstalk between neural cells in the developing brain remains unclear.

The aim of this study was therefore to explore whether E2 could protect the neonatal brain from excitotoxic brain injury through its effects on various glial cell types. To do this, we used both the model of ibotenate-induced brain injury mentioned above (Gressens et al., 1996) and various primary cell cultures.

Materials and Methods

All experiments were carried out in compliance with the ethical rules of the INSERM. The study and animal protocols were approved by the institutional review board (Bichat-Robert Debré ethics committee, Paris, France, approval number 2010-13/676-0010)

Animals and model of excitotoxic brain lesions

Rats (Sprague-Dawley, Janvier SAS, Le Genest-St-Isle, France) were housed in a temperature-controlled room (19°C–23°C) with a 12 h/12 h light/dark cycle and with food and water ad libitum. Ten µg Ibotenate (Tocris, Bristol, UK) diluted in Phosphate Buffered Saline (PBS) was injected intracerebrally (i.c.) in postnatal day 5 (P5) rat pups as previously described (Pansiot et al., 2010). Ibotenate is a glutamate analogue that activates both N-methyl-D-aspartate (NMDA) and metabotropic receptors, but does not activate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) or kainate receptors. Injections were performed using a 26-gauge needle and a 50 µl Hamilton syringe mounted on a calibrated microdispenser. The needle was placed in the frontoparietal area of the right hemisphere, 2.5 µm from the midline in the mediolateral plane, and 4 mm from the bregma in the rostrocaudal plane. Two 1 µl boluses of ibotenate were injected with an interval of 20 seconds between the two. The needle was left in place for an additional 20 seconds.

β-Estradiol injections

The animals received a first injection of E2 12 hours before the ibotenate injection, and a second at the same time as ibotenate. Pre/per exposure injection regimen was chosen for covering the initial phase of brain injury process. Beta-Estradiol (E2) (Sigma, E8875) was first dissolved in DMSO and then diluted in PBS with a final concentration of 5% DMSO, and

injected intraperitoneally (i.p., 100µl each injection) at 4, 40 or 400µg/kg. Control animals received similar volumes of vehicle (PBS 5% DMSO).

Dosage of estrogen

Animals were anesthetized with isoflurane and blood collected by cardiac puncture at 3 time points: 3h after the first E2 injection (H3), 12h after the first injection (H12) and 3h after the second injection (H15). The clotted blood was centrifuged (7 min, 4000 rpm) and the serum pooled to obtain 200µl per time point (n = 4-5), frozen and stored at -80°C for hormonal measurements. E2 concentrations were measured using the Estradiol HS RIA kit (Immunodiagnostic systems, Paris, France, OD-67031).

Determination of lesion size

In each treatment group, 10–15 animals were sacrificed by decapitation 5 days after ibotenate injection (i.e. on P10). The brain was harvested, immediately fixed in 4% formalin and kept in this solution for 5 days before paraffin embedding. Coronal sections 16 µm thick were cut and every third section was mounted and stained with cresyl violet. The size of cortical and white matter lesions can be defined by their extent along 3 orthogonal axes: the mediolateral axis (in the coronal plane), the radial axis (also in the coronal plane, from the pial surface to the lateral ventricle), and the fronto-occipital axis (in the sagittal plane). In previous studies using this model (Husson et al., 2002), we observed an excellent correlation among measurements along the 3 axes. Based on these findings, we cut serial sections of the entire brain in the coronal plane for this study. This permitted the accurate and reproducible determination of the fronto-occipital extent of the lesion in the sagittal plane. We used this measurement as an index of lesion volume. All animals, and either males or

females injected with 400µg/kg were analyzed separately for lesion size determination.

Immunohistochemistry

In each experimental group, we studied 8 to 10 pups on postnatal days P6, P10 depending on experiments.

Paraffin sections were immunolabeled with primary antibodies listed in Table 1 and labeling visualized using the streptavidin-biotin-peroxidase method and the chromogen diaminobenzidine, as previously described (Baud et al., 2003).

RNA purification and real-time PCR

Four hours after ibotenate injection, at least 8 animals per group (ibotenate +/- E2) were sacrificed by decapitation, the brains removed, a sample of the brain cortex and underlying white matter harvested at the i.c. injection site (ipsilateral) and at a similar location in the contralateral hemisphere, immediately snap-frozen in liquid nitrogen and stored at -80°C. Total RNA from the samples was extracted with the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). RNA quality and concentration were assessed by spectrophotometry using a NanoDrop™ apparatus (Thermoscientifique, Wilmington, DE, USA). 1 µg of total RNA was subjected to reverse transcription using the Iscript™ cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France). qRT-PCR was performed in duplicate for each sample on a CFX384 Real Time System (Bio-Rad), using SYBR Green Supermix (Bio-Rad) for 40 cycles. Amplification specificity was assessed by melting curve analysis. Primers were designed using Primer3 software and manufactured by Eurofins Genomics (Ebersberg, Germany). Primer sequences are summarized in Table 2. The expression of genes of interest was calculated relative to the expression of the reference

gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Analyses were performed using Bio-Rad CFX Manager 3.0.

Primary cell cultures

Primary oligodendrocyte progenitor cell (OPC) cultures

Mixed glial cells were isolated from the cortices of newborn (P0) Sprague–Dawley rat forebrains as previously described (Simonishvili et al, 2013). Briefly, tissues were enzymatically digested with trypsin and DNase I and then mechanically dissociated and plated in DMEM containing 10% FBS and 30% glucose with antibiotics. Mixed glial cells were grown in T75 flasks until they were confluent (10 days). Microglia were eliminated from the cultures by shaking the flasks on a rotary shaker for 1.5h at 260 rev./min and by using liposomal clodronate (Kumamaru et al., 2012). OPCs were isolated following an additional 18h of shaking. The percentage of microglial cells contaminating OPCs culture is below 1%. OPCs were seeded onto poly-D-lysine-coated 8-well Ibidi µ-slides (Biovalley) at a density 10^5 cells per well in a final volume of 250 µl/well. After 72h, OPC cultures were treated.

Primary neuron cultures

Cultured neurons were derived from the cerebral cortex of embryonic day (E) 17 rats. After dissection of the cortices and removal of the meninges, cortical cells were isolated as previously described (Baud et al., 2005). Cells were seeded either in 8-well Ibidi µ-slides (Biovalley) pre-coated with poly-L-lysine (Sigma) at 10^5 cells per well in a final volume of 250 µl/well. Arabinocytidine hydrochloride (AraC, 5 µm, Sigma, C1768) was added on the 4th Day In Vitro (DIV) to stop astrocyte proliferation. Cultured neurons were exposed to treatments on DIV 10.

Astrocyte/neuron co-cultures

Astrocytes were derived from the cerebral cortex of postnatal day 1 (P1) rat pups as

previously described (Degos et al, 2013). Microglial cells were separated from primary mixed glia cultures at DIV 14 by shaking vigorously. Neurons were prepared from the neocortex of E17 rat embryos as described above. One day before the plating of neurons, astrocytes were maintained in neuronal medium (DMEM) supplemented with Glutamax (Gibco, 35050-038), 5% FBS, N-2 and B-27 (Gibco, respectively 15502-048 and 17504-044) and 0.01% penicillin-streptomycin. Neurons were plated directly on the surface of confluent astrocytic monolayers at a density of 4.10^4 cells/cm^2. Co-cultures were exposed to treatments on DIV 10 for the neurons.

Treatments and cell cultures assessment

OPC cultures were treated with E2 (0.1µM) or vehicle, and then assessed after 24h exposure using immunocytochemistry. OPC cells were double-labeled using primary antibodies Olig2 and MBP (Table 1) and secondary antibodies coupled to the green fluorescence marker Alexa488 (Lifetechnology) or the red fluorescent marker cyanine 3 (Jackson Immunoresearch Laboratories, USA).

Neuronal cells cultures and astrocyte/neuron co-cultures were pre-treated by E2 (1-10µM or vehicle) 30 min before the excitotoxic insult. They were challenged during 5h using 300µM ibotenate and then assessed for cell death. To assess cell survival, we used Hoechst (Sigma, 94403), staining all nuclei, and 7-Aminoactinomycin D (Life Technologies, A1310), staining only non-viable cells.

Statistical analysis

All data are reported as means +/- S.E.M. Statistical analysis of all data was performed using GraphPad PRISM version 5.0 (GraphPad Software, San Diego, CA). The normal distribution of

all results was assessed with the D'Agostino and Pearson omnibus normality test. To compare two groups with a normal distribution, a t-test was performed whereas in the absence of a normal distribution, a Mann–Whitney U test was used. For more than 2 groups, a one-way ANOVA followed by a Dunnett's or Bonferroni post hoc test was performed.

Results

Effect of E2 on the size of excitotoxic lesions in the developing brain

E2 was injected twice, 12h apart, in P4 rat pups according to the protocol described in the Method section. No difference in mortality rate or growth was detected between experimental groups. Table 3 shows serum concentrations of E2 in rat pups 3 and 12 hours (H3 and H12) after the first injection and 3 hours after the second injection (H15). Following the injection of 4, 40 and 400 µg/kg of E2, serum concentrations were found to be roughly 8-, 50- and 280-fold higher respectively, compared to basal concentrations in P4 rat pups. At all E2 dosages, we observed a significant reduction in lesion size of the cortical plate on P10 following i.c. ibotenate injection in P5 rat pups (Figure 1A-D). The two highest dosages (40 and 400 µg/kg) were associated with a significant reduction in lesions size of the white matter (Figure 1A-D). The neuroprotective effect of 400 µg/kg E2 was found similar in males and females, in both cortex and white matter (Figure 1E, EF). Subsequent experiments were performed using 400 µg/kg of E2, because this dosage appeared to induce the maximal effect on brain damage and because it was in similar range than previously reported in the literature (Gerstner et al., 2007).

Neuroprotective effect of E2 on the developing brain: impact of E2 on microglial phenotype and cytokine synthesis in vivo.

Neuroprotection of the developing brain by E2 in our rat model has been next explored. Because neuro-inflammation is a phenomenon known to disrupt the maturation of the developing white matter and therefore myelination, E2 effect could thus be mediated by anti-inflammatory mechanisms.

The excitotoxic insult to the developing brain induced a dramatic and rapid (beginning 4h after i.c. injection) increase in the density of activated microglial cells (Husson et al., 2002). E2 was found to significantly reduce this microglial activation around the lesion site at P6 (Figure 2A). At P10, excessive microglial activation was no longer observed in response to ibotenate challenge. Interestingly, E2 treatment was found associated with a significant decrease in the density of activated microglial cells in both hemispheres within the developing white matter (Figure 2B).

To more precisely define the microglial activation, we next analyzed transcription levels for several markers of microglial phenotypes in response to ibotenate injection and E2 treatment. The ibotenate insult was found to induce within 4h a significant increased synthesis of pro-inflammatory cytotoxic markers (Lgals3, CD86, Cox2, iNOS) and immuno-modulatory markers (IL1rn, IL4ra), but reduced transcription of Arg1 and Mrc1, two repair/regeneration microglial markers (Figure 3). E2 treatment was found to reverse these changes for most of the markers and was therefore associated with modulation of the balance between microglial phenotypes.

Figure 4 summarizes the effects of E2 on the transcription of genes encoding cytokines and other molecules involved in inflammation and immune function in contralateral hemisphere to the excitotoxic insult (Veh and E2 groups) and in ipsilateral hemisphere to the ibotenate injection (Ibo+Veh and Ibo+E2 groups). E2 did not induce any significant changes in cytokine synthesis in the hemisphere contralateral to the ibotenate insult. In contrast, 4 hours after ibotenate injection, gene expression levels of various cytokines were found to be modified in brain samples harvested around the ipsilateral white matter and cortical lesion. E2 treatment was associated with a rapid (within 4h) and significant reversal of the ibotenate-induced change in the expression of genes encoding interleukin (IL) 6, IL1-beta and

prostaglandin D2 synthase (PTGDS) (Figure 4).

Altogether, these data strongly suggest that E2 is capable of reversing the inflammation associated with white matter and cortical lesions induced in the developing brain by excitotoxicity due to ibotenate, a glutamate agonist.

Neuroprotective effect of E2 on the developing white matter: impact of E2 on myelination and oligodendroglial maturation in vivo and in vitro

Besides its effect on neuroinflammation, we asked the question whether E2 could improve myelination and promote the oligodendroglial maturation.

After ibotenate challenge in vivo, E2 was found to be associated with a significant increase in the density of APC-positive mature oligodendrocytes both within the white matter adjacent to the injury site and in the ipsilateral cingulate white matter, compared to vehicle treatment (Figure 5A, 5C). In contrast, no detectable effect was observed regarding the density of all Olig2-positive oligodendrocytes in the same regions of interest (Figure 5B). In addition, myelin fiber density, as measured by labeling for MBP, was also found to be significantly increased following E2 treatment in two separate uninjured white matter areas (cingulum and lateral corpus callosum) (Figure 5D, 5E). This is in accordance with the downregulation of PDGRα gene transcription induced by E2, without significant changes in the expression of Sox10, Nkx2.2 or p27kip1 (Figure 5F).

In primary OPC cultures, 24h of exposure to E2 at very low concentration (0.1 µM) as compared to vehicle was associated with increased density of MBP-positive sheets without changing the density of Olig2-positive cells (Figure 5G-I). These data are consistent with those from in vivo experiments and suggest that E2 promotes oligodendrocyte maturation in both systems.

Role of astroglial cells in E2 neuroprotection in cerebral cortex and neurons

To better delineate the potential role of glial cells in E2-induced neuroprotection observed in vivo, we next investigated the effect of this hormone on astrocytes both in vivo and in vitro. We first hypothesized that the in vivo beneficial effect of E2 on the cortical plate could be mediated through astroglial cells. We observed that E2 cortical neuroprotection was not associated with reduced astroglial activation in response to cortical injury induced by ibotenate injection (Figure 6A). In addition, we found that E2 promoted upregulation of TGFβ1 and GDNF gene expression when cortex was subjected to excitotoxic challenge (Figure 6B). These growth factors were known to play a role in astrocytes-induced survival of neurons (Platania et al., 2005; Dhandapani et al., 2005). We next replicated in vitro our in vivo model of excitotoxic injury by subjecting during 5h neuronal cells alone to 300 µM of ibotenate, a concentration known to induce significant reduction in neuronal survival (Degos et al., 2013). E2, at any concentrations used (1, 10 µM) after 30 min pretreatment duration was not capable of significantly reducing ibotenate-induced neuronal death in culture (Figure 6C). We therefore asked whether the cortical neuroprotection observed in vivo was indeed related mediated by astrocytes in response to ibotenate. We used co-cultures in which neurons were in contact with a confluent astrocytic monolayer. Under these experimental conditions, significant neuroprotection has been already observed without E2 (Figure 6C). The addition of E2 (1-10 µM) to the co-cultures 30 min before excitotoxic challenge was associated with full and much more pronounced protection against neuronal necrosis induced by ibotenate and assessed using 7-aminoactinomycin D (Figure 6C, D).

Discussion

In this study, we demonstrate that E2 exposure significantly reduces lesion size in both the cortical plate and white matter in rat pups subjected to excitotoxic brain injury. While some reports have already shown that estradiol is partially protective in a neonatal model of brain damage induced by kainic acid (Mc Carthy, 2008; Hilton et al., 2004), the precise mechanisms have remained unclear. Here, we have better defined the main effects of E2 in the developing brain, in vivo and in vitro, and provide new insight into the cellular crosstalk that mediates the response to E2 treatment:

- first, E2 has significant effect on the acceleration of oligodendrocyte maturation in the developing brain,
- second, the E2-mediated neuroprotection observed in rat pups was associated with significant changes in microglial phenotype in response to brain injury.
- third, astrocytes appear to play a key role in mediating E2 neuronal effect.

The developing brain expresses high levels of receptors for estrogens derived from the maternal circulation, fetal gonads, and local synthesis in the brain. Several animal studies have demonstrated that estradiol of both gonadal and brain origin is neuroprotective in adults (Arevalo et al., 2015). In particular, studies in female rodents have shown that decreasing plasma estradiol concentrations by ovariectomy exacerbates brain damage under neurodegenerative conditions (Azcoitia et al., 1999; Azcoitia et al., 2011; Yue et al., 2005; Ding et al., 2013; Overk et al., 2012). Estradiol could be also neuroprotective in humans, as aging and menopause are thought to potentiate age-related cognitive impairment and Alzheimer's disease (Scott et al., 2012).

In vitro studies investigating the mechanism of action of estradiol have shown that it promotes the survival of mature neurons even in the absence of glial cells (Chowen et al., 1992; Valles et al., 2008). In our study, however, no concentration of E2 tested had any effect on primary neuronal cultures subjected to ibotenate injury but neuroprotective effects on neurons were observed when astrocytes and neurons were co-cultured. These findings were in accordance with other reports (Dhandapani and Brann, 2003, 2007). Furthermore, TGFβ1 and GDNF gene expression was found to be upregulated by E2, consistently with the central role of astrocytes in E2-mediated neuronal protection against excitotoxic insult as previously shown (Platania et al., 2005; Dhandapani et al., 2003, 2005). Astrocytic glial cells derived from rat neonatal cortex have been shown to express both estrogen receptors (ER) α and β proteins in both the nuclear fractions and plasma-membrane fractions (Chaban et al., 2004). ERs are also expressed in the oligodendroglial plasma membrane and decrease with maturation (Arvanitis et al., 2004; Gerstner et al., 2009). Because brain injury induces the synthesis of estradiol in these cells, ERs likely play a key role in this estradiol-mediated neuroprotection. Further studies are ongoing to explore endogenous steroidogenesis and the pattern of expression of aromatase, the enzyme responsible for the conversion of testosterone into estrogens, in dividing neural precursors harvested from the developing rat brain. Indeed, increased aromatase activity in astrocytes, which do not express this enzyme under basal conditions in adult rodents, appears to play a key role in the neuroprotective effects of estradiol both in animals and humans (Garcia-Segura et al., 1999; Azcoitia et al., 2001). This mechanism needs to be further explored in the developing brain, in order to understand the crucial involvement of astrocytes in neuroprotection in vitro in our study.

The role of glial cells in the neuroprotective properties of estradiol has been mainly described in in vitro systems, in which estradiol appears to act on glial and endothelial cells to maintain the function of the neurovascular unit in vivo, to control neuroinflammation and to maintain the function of neuronal circuits (Arevalo et al., 2010; Spence et al., 2013; Dhandapari and Brann, 2007). Here we demonstrate that estradiol is also able to enhance oligodendrocyte maturation and myelination in the developing brain, a key phenomenon associated with reduced brain damage as previously reported in several models of neonatal brain injury (Van Steenwinckel et al., 2014). The protective effect of E2 against hypoxic-ischemic injury to developing oligodendrocytes and white matter has been already reported (Gerstner et al., 2009), but not following excitotoxic injury in the immature brain.

Not surprisingly, E2 also demonstrated anti-inflammatory properties in the present study in a context of excitotoxic insult. Indeed, several reports have shown that both 17β-E2 and selective estrogen receptor modulators could mediate anti-inflammatory pathways in microglial cells in other models (Tapia-Gonzalez et al., 2008; Bruce-Keller et al., 2000). 17β-E2 has previously been shown to prevent the production and secretion of pro-inflammatory cytokines such IL-1β, IL-6, and TNFα, through the inhibition of NFκB signaling and the transcription of pro-inflammatory genes (Nadkarni and McArthur, 2013). Here, we found that neuroinflammation induced by the excitotoxic insult was one of the main targets of E2 treatment, leading to both a downregulation of genes encoding pro-inflammatory molecules and a reduction in the density of activated microglia. A recent study has reported that sex steroids could regulate hypoxia-induced inflammatory responses and the microglial phenotype switch in rat primary microglia in vitro (Habib et al., 2014). Our study refines the microglial phenotype in response to E2 treatment in vivo, and reveals that E2 tilts the balance in favor of repair and immunomodulatory factors. In addition, Favrais et al. have

demonstrated that neuroinflammation induces a blockade in the maturation of the oligodendroglial lineage (Favrais et al., 2011). The attenuation of neuroinflammation could therefore promote oligodendrocyte maturation in treated rat pups.

In addition to the effect of E2 on brain lesion size, neuroinflammation and cell lineages, long-term behavioral studies are also needed to confirm functional benefit of this neuroprotective strategy. A recent study demonstrated that estradiol improves functional outcome following spinal cord injury in rat (Mosquera et al., 2014). In stroke, E2 induces increases in neurogenesis and contributes to functional recovery (Li et al., 2011).

Although epidemiological and animal studies point to estrogen as an important factor in neuroprotection, future clinical translation might be not simple for several important reasons. First, supra-physiological levels used here and elsewhere for inducing neuroprotective effect in rodents were not compatible with clinical translation. Second, the functions of estrogens are intimately associated with those of progesterone and androgens (Chowen et al., 2000), making it difficult to consider each independently with respect to neuroprotection. Finally, because the role of estrogens in neuroprotection may be indirect, through actions on other organs which then impinge upon the health of the brain. The therapeutic role of estrogens in neuroprotection could benefit from the development of selective estrogen receptor modulators (SERMs) that preferentially target one estrogen-sensitive tissue over others (Merchenthaler et al., 2003; Azcoitia et al., 2011; Arevalo et al., 2012). While the specific actions of SERMs in the CNS need to be evaluated, they may ultimately prove useful in targeting the neuroprotective effects of estrogen analogs without activating neuroendocrine functions.

In conclusion, E2 provides significant neuroprotection both in the cortical plate and the white matter in neonatal rats subjected to excitotoxic brain injury. This effect is implemented through the interactions of multiple glial cell types in the injured brain (Johann and Beyer, 2013). Further in vivo studies are needed to better understand the signaling pathways involved in the response of glial cells to estrogenic compounds and to assess their long-term behavioral effect before their translation to the clinical setting. In this respect, SERMs could constitute an interesting and novel approach to modulate microglial activation and CNS inflammation in neonates at risk of brain damage.

Tables and Figure legends

Table 1: Antibodies and markers used in the experiments.

Table 2: Primer sequences used in the experiments.

Table 3: Serum E2 concentrations 3, 12 and 15h after the onset of the treatment (two injections were carried out, at H0 and H12) (values are expressed in pg/mL).

Figure 1: E2 confers neuroprotection against excitotoxic brain lesions in neonatal rats.

A, B. Quantitative analysis of lesion size (fronto-occipital extent) in the cortical plate (A) and white matter (B) following the i.c. injection of ibotenate (ibo) in P5 rat pups (n=10-15) with and without E2 pre-exposure (2 doses of 4, 40 or 400 µg/kg were injected 12h and at the time of ibotenate challenge).

*: $p<0.05$ and ***: $p<0.001$ for comparisons between the treated groups and Veh group using one-way ANOVA with Dunnett's correction.

C, D. Cresyl-violet–stained sections showing brain lesions induced by ibotenate intra-cerebrally injected on P5 and observed on P10. E2 reduced the size of the ibotenate-induced cortical lesion (red arrow) and white matter cyst (blue arrow). Bar = 50 µm; LV: lateral ventricle.

E, F. Quantitative analysis of cortical (E) and white matter (F) lesion size following the i.c. injection of ibotenate in P5 females and males (n=9 in each group) with and without E2 pre-exposure (2 doses of 400 µg/kg). *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ for comparisons between Veh and E2 groups, using a one-tailed t-test.

Figure 2: Impact of E2 treatment on excitotoxic white matter injury in rat pups.

Quantitative analysis of the density of activated microglia in the white matter surrounding the cystic excitotoxic lesion (ipsilateral to ibotenate injection) and in the contralateral white matter. Cell densities were evaluated by tomato lectin labeling at 24h (A) and ED1 immunoreactivity at 5 days (B) in animals treated either by 400 µg/kg E2 or vehicle; n=8-10 in each group.

*: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ for comparisons between contralateral hemisphere in vehicle-treated animals and the other experimental groups using one-way ANOVA with Dunnett's correction. ##: $p<0.01$ and ###: $p<0.001$ for comparisons between the two groups indicated, using a one-tailed t-test.

Figure 3: Early impact of E2 on gene expression levels of various microglial phenotypic markers in P5 rat pups 4h after ibotenate insult. Veh: contralateral hemisphere in vehicle-treated animals; E2: contralateral hemisphere in animals treated by 400 µg/kg E2; ibo+Veh: ipsilateral hemisphere in vehicle-treated animals; Ibo+E2: ipsilateral hemisphere in animals treated by 400 µg/kg E2. Lgals3, CD86, Cox2 and iNOS are pro-inflammatory cytotoxic markers, Mrc1 and Arg1 are repair/regeneration markers, IL1rn and IL4ra are immunomodulatory markers; n=8 in each group.

*: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ for comparisons between contralateral hemisphere in vehicle-treated animals and the other experimental groups using one-way ANOVA with Dunnett's correction. #: $p<0.05$, ##: $p<0.01$ and ###: $p<0.001$ for comparisons between the two groups indicated, using a one-tailed t-test.

Figure 4: Early impact of E2 on gene expression levels of various cytokines and prostaglandins-related proteins.

Quantitative PCR were performed in P5 rat pups 4h after ibotenate insult. Veh: contralateral hemisphere in vehicle-treated animals; E2: contralateral hemisphere in animals treated by 400 µg/kg E2; ibo+Veh: ipsilateral hemisphere in vehicle-treated animals; Ibo+E2: ipsilateral hemisphere in animals treated by 400 µg/kg E2; n=8 in each group.

*: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ for comparisons between contralateral hemisphere in vehicle-treated animals and the other experimental groups using one-way ANOVA with Dunnett's correction. #: $p<0.05$ and ##: $p<0.01$ for comparisons between the two groups indicated, using a one-tailed t-test.

Figure 5: Effect of E2 on oligodendrocyte maturation with or without an excitotoxic challenge.

A-C: Quantitative analysis of the density of APC-positive (A) and Olig2-positive (B) cells in two regions ipsilateral to the ibotenate injection: close to the white matter damage (WMD) and within the cingulate white matter (CWM). Regions analyzed are indicated by boxes superposed on cresyl-violet–stained sections showing brain lesions induced by i.c. ibotenate injected on P5 and observed on P10 (C). Bar = 50 µm; LV: lateral ventricle; n=8-10 in each group. *: $p<0.05$, **: $p<0.01$, for comparisons between Veh and E2 groups, using a one-tailed t-test.

D: Quantitative analysis of the optical density of myelinated fibers in the cingulate white matter and lateral corpus callosum in P10 rat pups without excitotoxic challenge; n=8-10 in each group. *: $p<0.05$ for comparisons between Veh and E2 groups, using a one-tailed t-test.

E: Representative photomicrographs of MBP-positive fibers in the cingulate white matter on P10 in rat pups treated with 400 µg/kg of E2 on P5 (+E2) compared to rat pups injected with Vehicle (+Veh), without ibotenate challenge. Bar = 50 µm.

F: Quantitative analysis of gene expression levels of PDGFRα, Sox10, Nkx2.2 and p27kip1 in P5 rat pups 4h after treatment with 400 µg/kg E2 (black bars) compared to untreated controls (Veh, white bar); n=8 in each group.

**: $p<0.01$ for comparisons between the treated groups and Veh, using one-way ANOVA with Dunnett's correction.

G-I: The density of Olig2-positive cells and ratio of the optical density (OD) of myelin basic protein (MBP) to the density of Olig2-positive cells after 24h of exposure to either vehicle or 0.1 µM E2 in oligodendroglial precursor cells in vitro.

**: $p<0.01$ for comparisons between the two groups indicated, using a one-tailed t-test.

Figure 6: Role of astrocytes in the beneficial effects of E2 on neuronal cells subjected to an excitotoxic insult in vivo and in vitro.

A. Quantitative analysis of the density of GFAP-positive cells in the somato-sensory cortex in ipsilateral (ibotenate injured) and contralateral (no injury) hemispheres. No difference was observed between Veh and E2 groups in each hemisphere, using a one-tailed t-test; n=8-10 in each group.

B. Quantitative analysis of the transcription of genes involved in astrocytic-induced neuroprotection in cortical samples from P10 rat pups after ibotenate insult. Veh: contralateral hemisphere in vehicle-treated animals; E2: contralateral hemisphere in animals treated by 400 µg/kg E2; ibo+Veh: ipsilateral hemisphere in vehicle-treated animals; Ibo+E2: ipsilateral hemisphere in animals treated by 400 µg/kg E2; n=8 in each group.

*: p<0.05, **: p<0.01 and ***: p<0.001 for comparisons between the two groups indicated, using one-way ANOVA with Dunnett's correction.

C. Three hundred µM ibotenate exposure during 5h was found to induce significant neuronal cell death. E2 has no effect on ibotenate-induced cell death in neurons only (***: p<0.001, using one-way ANOVA with Bonferroni correction) significant neuroprotection has been observed when using a two-layer co-culture system in which neurons were in contact with astroglial cells (##: p<0.01, comparison between neurons only and co-cultures subjected to 300 µM ibotenate, without E2). This neuroprotection was found significantly more pronounced when the system was exposed to E2 (1 and 10 µM). §§§: p<0.001 for comparisons between groups exposed to E2 and without E2 using one-way ANOVA with Dunnett's correction.

D. Necrotic cell death in the neuronal layer of the co-culture system as seen using 7-aminoactinomycin D, which stains necrotic nuclei red. Bar = 100 µm.

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